SUMMARY OF THE INVENTION

An object of the present invention is to provide CNTF and CNTF-related proteins, collectively referred to herein as CNTF proteins, for the treatment of diseases or disorders including, but not limited to, obesity and diabetes.

A further object of the present invention is to provide a method for administering CNTF or CNTF-related proteins and maintaining biological activity. A preferred embodiment of this invention is the administration of CNTF or a CNTF related protein to the nasal or respiratory system of a mammal to produce an increase in the level of the protein in the systemic blood circulation of the mammal. A particularly preferred embodiment comprises the administration of the modified CNTF molecule, designated herein as AX-15, to the nasal passages of a patient for the treatment of obesity or diabetes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 - Alignment of CNTF protein sequences. A. Human, rat, rabbit, mouse and chicken (Leung, et al., 1992, Neuron 8:1045-1053) sequences. Dots indicate residues found in the human sequence. B. Modified CNTF molecules showing human CNTF amino acid residues (dots) and rat CNTF (residues shown). The name of the purified recombinant protein corresponding to each sequence is shown on the left.

Figure 2 - Mobility of human, rat and several modified CNTF molecules on reducing SDS-15% polyacrylamide gels. Purified recombinant proteins were loaded as indicated. Markers of the indicated MW were loaded on lane M.

Figure 3 - Effects of Axokine-15 (AX-15) in normal mice. Normal C57BL/6J mice were injected subcutaneously daily for 6 days with either vehicle or AX-15 at 0.1 mg/kg, 0.3 mg/kg, or 1.0 mg/kg. Percent change in body weight in AX-15-treated versus vehicle-treated controls is shown.

The above observations led to a directed effort to identify the region on the CNTF molecule responsible for these differences. This method involved the exchange, by genetic engineering methods, of parts of the human CNTF sequence with the corresponding rat CNTF sequence and vice versa. To achieve this, advantage was taken of restriction sites that are common to the two CNTF genes and unique in their corresponding expression vectors. When necessary, such sites were engineered in one or the other of the two genes in areas that encode the same protein sequence. With this approach, expression vectors were obtained for each of the modified proteins shown in Figure 1A and 1B. After expressing and isolating the individual proteins to at least 60% purity, their properties, as compared to those of human and rat CNTF, were determined.

Because the electrophoretic mobilities of human and rat CNTF differ significantly, the effect of each amino acid substitution was monitored initially by making a determination of the effect of such change on the mobility of the protein. As described herein, electrophoretic mobility data indicated that all of the modified human CNTF molecules that migrated to the same position as rat CNTF had the single amino acid substitution Gln63Arg (Q63R), in which glutamine at position 63 is replaced with arginine.

CNTF is characterized by its capacity to support the survival of dissociated ciliary neurons of E8 chick embryos. By this criterion, purified recombinant rat CNTF is as active as the native protein from rat, but four times more active than recombinant human CNTF (Masiakowski, et al., 1991, J. Neurosci. 57:1003-1012 and in International Publication No. WO 91/04316, published on April 4, 1991). The same assay was utilized to determine the biological activity of the altered molecules prepared as described above. As described herein, all modified CNTF molecules that had the Q63R substitution exhibited an increased ability to support the survival of ciliary ganglion neurons as compared to the parent human CNTF protein. Such results indicated a strong correlation between alteration electrophoretic mobility and

The expression vectors pRPN33, which carries the human CNTF gene and pRPN110 which carries the rat CNTF gene are nearly identical (Masiakowski, et al., 1991, J. Neurosci. 57:1003-1012 and in International Publication No. WO 91/04316, published on April 4, 1991.)

Plasmid pRPN219 was constructed by first digesting pRPN33 with the restriction enzymes Nhe1 plus Hind3 and gel purifying the 4,081 bp fragment. The second, much smaller fragment which codes for part of the human CNTF gene was subsequently replaced with an 167 bp Nhe1-Hind3 fragment that was obtained by PCR amplification from the rat gene using the primers RAT-III-dniH: 5' ACGGTAAGCT TGGAGGTTCTC 3' (SEQ ID NO. 18) and RAT-Nhe-I-M: 5' TCTATCTGGC TAGCAAGGAA GATTCGTTCA GACCTGACTG CTCTTACG 3' (SEQ ID NO. 19)

Plasmid pRPN228 was constructed in the same manner as pRPN219, except that the 167 bp replacement fragment was amplified using the DNA primers Rat-III-dniH-L-R : 5' AAG GTA CGA TAA GCT TGG AGG TTC TCT TGG AGT CGC TCT GCC TCA GTC AGC TCA CTC CAA CGA TCA GTG 3' (SEQ ID NO. 20) and Rat-Nhe-I: 5' TCT ATC TGG CTA GCA AGG AAG 3' (SEQ ID NO. 21)

Plasmids pRPN186, pRPN187, pRPN188, pRPN189, pRPN192, pRPN218, and pRPN222 were generated by similar means or by direct exchange of DNA fragments using the unique restriction sites shown in Figure 1A and 1B

The identity of all plasmids was confirmed by restriction analysis and DNA sequencing.

Protein Purification

Induction of protein synthesis, selective extraction, solubilization and purification from inclusion bodies were as described for rat and human CNTF (Masiakowski, et al., 1991, J. Neurosci. 57:1003-1012 and in International Publication No. WO

Competition binding experiments with the other modified CNTF proteins shown in Figure 1 also demonstrated that proteins having R63 displayed the biological activity of rat CNTF, whereas proteins having Q63 displayed the binding properties of human CNTF (data not shown). These results indicate that the single amino acid substitution (Q63->R) is sufficient to confer to human CNTF the receptor binding properties characteristic of rat CNTF.

Example 3 - Construction of AX-15 Expression Plasmid pRG643

The expression plasmid pRG632 is a high copy plasmid that encodes ampicillin resistance and the gene for human CNTF-C17A,Q63RΔC13 (also referred to herein as either AX-1 or AX-13) with a unique Eag I restriction enzyme recognition sequence 3′ to the stop codon. This plasmid was used to construct a human CNTF mutation C17A,Q63R,ΔC15 (designated AX-15) by PCR amplification of a 187 bp BseR I-Eag1 DNA fragment that incorporates the ΔC15 mutation. The 5′ primer {ΔC15- 5′ (5′-CCAGATAGAGGAGTTAATGATACTCCT-3′)} [SEQ ID NO: 22] encodes the BseR I site and the 3′ primer ,ΔC15-3′ {(5′-GCGTCGGCCGCGGACCACGCTCATTACCCAGTCT GTGAGAAGAAATG-3′)} [SEQ ID NO: 23] encodes the C-terminus of the AX-15 gene ending at Gly185 followed by two stop codons and an Eag I restriction enzyme recognition sequence. This DNA fragment was digested with BseR I and Eag I and ligated into the same sites in pRG632. The resulting plasmid, pRG639, encodes the gene for AX-15 (human CNTF C17A,Q63R, ΔC15). The ΔC15 mutation was then transferred as a 339 bp Hind III-Eag I DNA fragment into the corresponding sites within pRG421, a high copy number expression plasmid encoding the gene for kanamycin resistance and human CNTF C17A,Q63R,ΔC13. The resulting plasmid, pRG643, encodes the gene for AX-15 under transcriptional control of the lacUV5 promoter, and confers kanamycin resistance. The AX-15 gene DNA sequence was confirmed by sequence analysis.

was loaded onto a 50 L S-100 Sephacryl (Pharmacia) sizing column and eluted with 250 L of the same 5.0 mM sodium phosphate buffer, pH 8.0-8.3. Peak material exceeding 40% maximum A_{280} on the ascending portion of the peak and 40% of the maximum A_{280} on the descending portion of the peak was pooled. The pooled AX-15 protein was filtered through Millipak 0.22 µm filters and stored at -80°C prior to dispensing or formulation. The amino acid sequence of AX-15 produced follows. Alternatively, one could produce a sequence which contains a methionine residue before the initial alanine.

SEQ ID NO: 16

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          9          19          29          39          49          59
     *    *     *    *     *    *     *    *     *    *     *    *
 AFTEHSPLT PHRRDLASRS IWLARKIRSD LTALTESYVK HQGLNKNINL DSADGMPVAS

         69          79          89          99         109         111
     *    *     *    *     *    *     *    *     *    *     *    *
TDRWSELTEA ERLQENLQAY RTFHVLLARL LEDQQVHFTP TEGDFHQAIH TLLLQVAAFA

        129         139         149         159         169         179
     *    *     *    *     *    *     *    *     *    *     *    *
YQIEELMILL EYKIPRNEAD GMPINVGDGG LFEKKLWGLK VLQELSQWTV RSIHDLRFIS

     *
SHQTG
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SEQ ID NO: 17

METHIONINE+

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          10          20          30          40          50          60
     *    *     *    *     *    *     *    *     *    *     *    *
MAFTEHSPLT PHRRDLASRS IWLARKIRSD LTALTESYVK HQGLNKNINL DSADGMPVAS

         70          80          90         100         110         120
     *    *     *    *     *    *     *    *     *    *     *    *
TDRWSELTEA ERLQENLQAY RTFHVLLARL LEDQQVHFTP TEGDFHQAIH TLLLQVAAFA

        130         140         150         160         170         180
     *    *     *    *     *    *     *    *     *    *     *    *
YQIEELMILL EYKIPRNEAD GMPINVGDGG LFEKKLWGLK VLQELSQWTV RSIHDLRFIS

     *
SHQTG
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